PATENT

VANM159.001AUS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Remacle et al.

Group Art Unit 1631

#13
P.Lunkel
3/27/02

Appl. No : 09/574,626

Filed : May 19, 2000

For : METHOD FOR THE IDENTIFICATION AND/OR THE QUANTIFICATION OF A TARGET COMPOUND OBTAINED FROM A BIOLOGICAL SAMPLE UPON CHIPS

Examiner : S. Zhou

DECLARATION OF José REMACLE,

Assistant Commissioner for Patents

Washington D.C. 20231

Dear Sir:

I, José Remacle, declare as follows :

1. I am a one of the inventors in the US patent application 09/574,626.

2. I have been working in the field of biochemistry and cell biology for many years. My Curriculum Vitae is enclosed to this Declaration.

3. I have read the content of the US patent no. 6,027,890 issued on February 22, 2000 in the name of Van Ness et al. Said document describes the use of streptadin / peroxidase conjugate for testing the presence of a binding by

hybridization of biotinylated DNA sequences upon corresponding oligonucleotide sequences bound upon the surface of a solid support. The activity of said peroxidase results in the formation of a precipitate upon the solid support. Said US patent suggests that said solid support could be a micro-array.

However, said US patent does not provide any information regarding the reliability and the precision of the detection.

4. I present hereafter experimental data performed in my laboratory. Said comparative results were obtained by using the method proposed in the US patent no. 6,027,890 compared to the results obtained with the method according to the present invention. The materials and methods used are described in the following annex.

The first figure presents a quantitative comparison of colorimetric detection systems on micro-arrays using antibiotin linked to gold or streptavidin linked to gold, which allows thereafter the formation of a silver precipitate compared to the use of streptavidin linked to peroxidase.

Said figure shows that the method according to the invention results in a detection of a metallic precipitate which is 1000 times more sensible than a similar detection of a method using a precipitate resulting from the enzymatic reaction of peroxidase. Indeed, a spotting solution of 0.1 nm concentration in liotinylated capture DNA was the limit of sensitivity with the antibiotin-gold and silver precipitation while the limit of detection for the peroxidase based precipitate was only 100 nm.

5. The formation of a metallic precipitate presents also advantageous characteristics because it can be rapidly (in a few minutes) detected. A silver precipitate was obtained after 10 minutes while a peroxidase reaction was performed for more than 3 hours before a precipitate can be characterised.

6. The formation of a metallic precipitate can be more easily detected by different detection means due to its refracture/defracture and possible conductive and/or magnetic characteristics.

7. The method according to the invention is also based upon a reaction leading to the advantageous formation of a precipitate at the location of the bound target compound upon its corresponding capture molecule which means lower than a few micrometers from the location of the bound target compound.

   Such characteristics render the method according to the invention well-suited for the detection of biological molecules upon high or even very high density micro-arrays.

8. As an expert in this field, I can state that the state of the art does not teach or suggest that a method based upon the precipitation of a metal deposit will be so extremely efficient and will be characterised by such high sensibility which allows its use in high density micro-arrays.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on

information and belief are believed to be true; and further that these statements were made with the knowledge that wilful and false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application and any patent issued thereon.

Respectfully submitted,

Dated: 19 November 2001

By: _____

José REMACLE

Protocol for comparison of colorimetric detection method on DNA microarrays

Biotinylated DNA were spotted at different concentrations (from 100nM to 0.001nM) on plastic support.
After spotting, supports were washed with SDS 0.2% 1 min at room temperature, then 1 min in water and finally, 3 min in boiling water for denaturation of DNA.
Supports were washed then in 10mM maleate buffer pH7.5 NaCl 15mM Tween 0,1 % (Washing buffer) 4X1min at room temperature.
Three supports were then incubated with Blocking buffer (100 mM maleate buffer pH 7.5 ,NaCl 150 mM, Milk powder 0,1%) containing a conjugate : one is antibiotin-gold labeled diluted 100X, second is streptavidin gold (stav-gold) diluted 100X and the third conjugate is streptavidin-HRP (stav-HRP) diluted 1000X). this incubation is at room temperature for 45 min.
Supports were then washed 5X for1 min in washing buffer.
Support 1 incubated with antibiotin-gold was then incubated 8 min in Silver Blue Solution (AAT, Namur, Belgium), then rinsed in water and dried.
Support 2 incubated with stav-gold was then incubated 10 min in Silver Blue Solution, then rinsed in water and dried.
Support 3 incubated with stav-HRP was then incubated 3 hours in diaminobenzidine 0,2mg/ml in 0.1M Tris HCl buffer pH 7.4 containing 0,25% H2O2, then rinsed in water and dried.

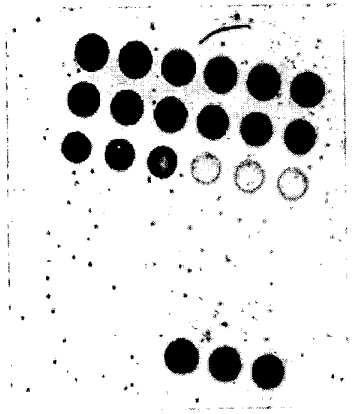
Conlusion

The limit of detection for the antibiotin/gold -silver precipitate is obtained with a solution of 0.1 nM DNA and with a is 3nM.for the streptavidin/gold-silver precipitate However when the peroxidase is used for production of a precipitate, a detection limit is obtained with a solution of 100nM even with a precipitation reaction going on for 3 h.
We can conclude that the nanogold-silver precipitate is around 1000 times more sensitive than the peroxidase precipitate. This is unexpected results since both precipitates are the result of catalytic reactions and there was no reason to suspect the silver to be so sensitivve compared to the peroxidase. In an absolute level, the detection of a spotting solution of 0.1nmM DNA represents an amount of around 5x10-20 mole since around 0.5nl of solution are used for each spot. This is a real unexpected sensitivity.

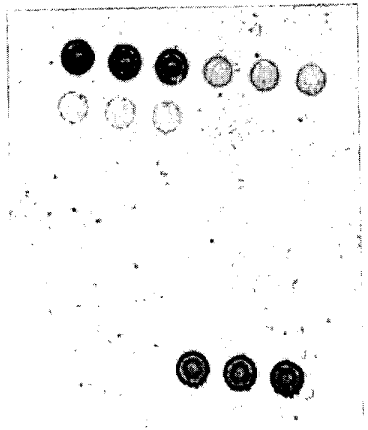
Comparison of colorimetric detection systems on microarrays

100nM	● ● ● ● ● ●	30nM
10nM	● ● ● ● ● ●	3nM
1nM	● ● ● ● ● ●	0,3nM
0,1nM	● ● ● ● ● ●	0,03nM
0,01nM	● ● ● ● ● ●	0,003nM
0,001nM	● ● ● ● ● ●	Negative ctl
Mec A	● ● ● ● ● ●	100nM

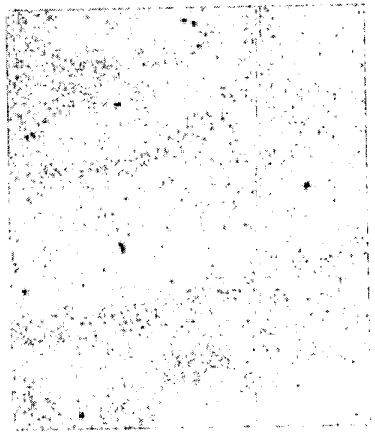
The numbers give the concentrations of the spotting capture solutions . They are spotted in triplicates.



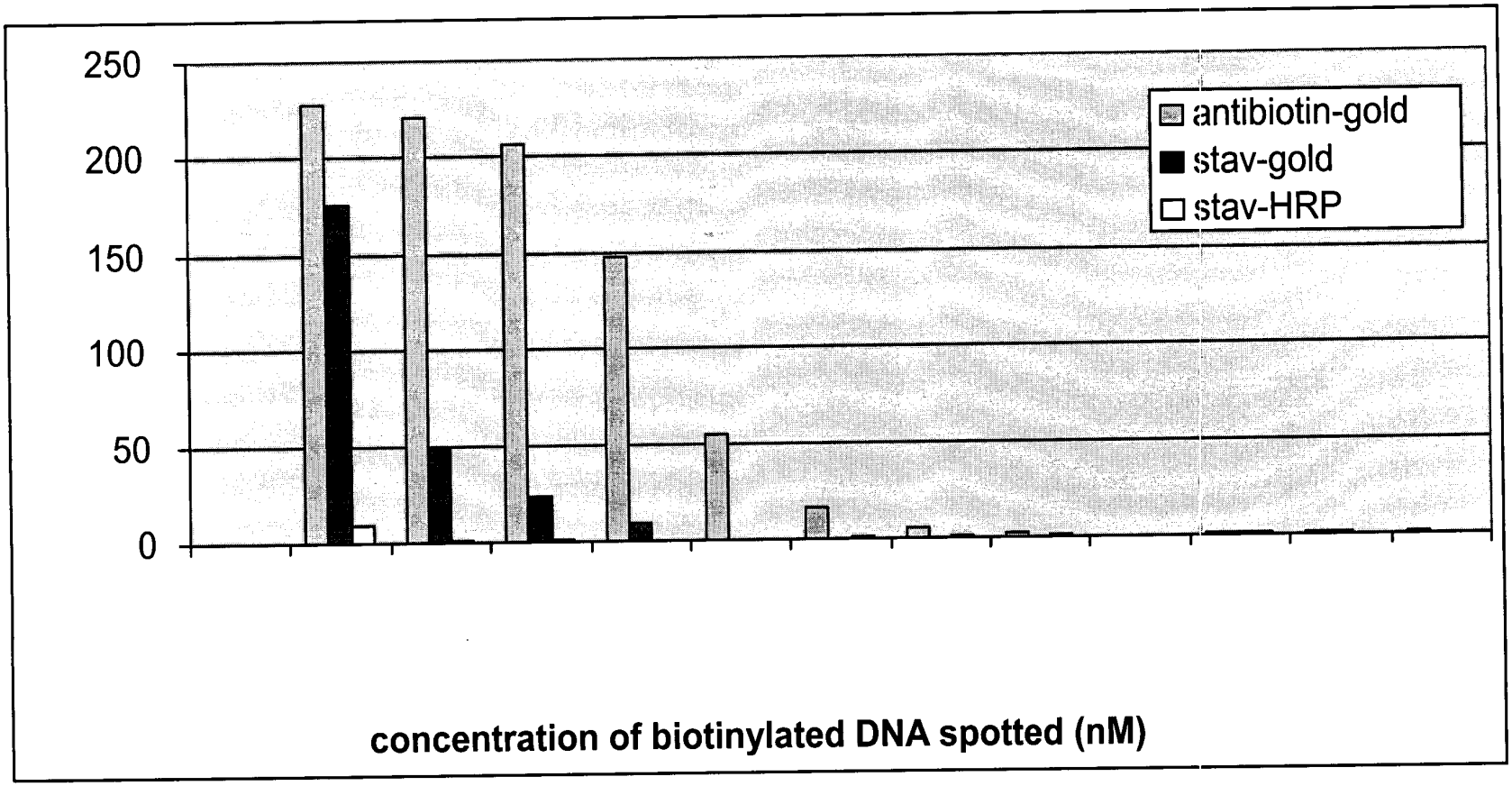
Antibiotin-gold
+
Silver Blue 8 min



Streptavidin-gold
+
Silver Blue 10min



Streptavidin-peroxidase
+
H2O2 and DAB 3 hours


250
200
150
100
50
0
antibiotin-gold
stav-gold
stav-HRP
concentration of biotinylated DNA spotted (nM)

CURRICULUM VITAE

REMACLE, José, A. L.

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Tel : 32-(0)81-724123 (Office) Fax : 32-81-724135
Email : jrem@biocell.fundp.ac.be

PERSONAL INFORMATION :

Place and date of birth : : Nassogne (Belgium), on the 31st August, 1946
Marital status : married
Nationality Belgian

DEGREES

- *Bachelor of Chemistry with maxima cum laude, 1970*
  Université Catholique de Louvain, Belgium.
- *Ph.D. in Sciences, Biochemistry, with maxima cum laude, 1973*
  Université Catholique de Louvain, Belgium.
  Directeurs de thèse : Profs H. Beaufay and A. Trouet.
  Laboratoire de Chimie Physiologique, Prof. C. de Duve.

POSITIONS

1970 - 1971 : *Junior research of the National Fondation for Scientific Research (F.N.R.S.)*
1971 - 1974 : *Research assistant of the F.N.R.S.*
1973 - 1974 : Fellowship of "Belgian American Educational Foundation" (Bourse C.R.B.)
1974 : Research fellowship of the European Molecular Biology Organization (E.M.B.O.)
1974 *Associate professor* Facultés Universitaires Notre-Dame de la Paix, Namur.
1980: *Professor* Facultés Universitaires Notre-Dame de la Paix, Namur
Director of the Laboratory of Cellular Biochemistry.
1985: *Full Professor, with tenure*
1992: *Visiting Scientist* University of Maryland, Baltimore County Campus

AWARDS

1968 : Prix de "l'Union Carbide European Research Associates"
1973 : Bourse William Hallam Tuck, of the fondation Francqui
1984 : Prix Vander Stricht de la Fondation André Vander Stricht.
1992 : Senior Research Scholar at the University of Maryland, Baltimore for 1992-1995.

PROFESSIONAL EXPERIENCE

Research stage at the Rockefeler University, Prof. C. de Duve, from July to September 1973.
Post-doctoral research at the University of California, San Diego, U.S.A., from September 1973 to August 1974, in the laboratory of Prof. S. J. Singer.
Scientific mission of 4 months at the Biochemical Engineering Department of the University of Maryland in Baltimore,Laboratory of Prof G. Rao, in 1992.

Scientific mission at the Biochemical Engineering Department of the University of Maryland at Baltimore as Senior Research Scolar in March-April 1993.

SCIENTIFIC RESPONSABILITIES :

Head of the laboratory of cellular biochemistry

Actual composition (1997)
6 PhDs in Science full research
12 PhDs Students
6 Graduate full research
5 Under-graduate students
6 technicians

Students and researchers already formed

Director of 16 PhDs Thesis passed from 1981 to 1997.
Director of 74 graduate students from 1974 to 1997.

RESEARCH CONTRACTS

Reseach Contracts with Industries

30 research contracts with Laboratoires Dausse, Synthélabo, Solvay-Biotec, Compagnie des développements agro-alimentaires (CDA), Kali-Chemie Pharma, La Floridienne, CELAC, laboratoires Oberval, Laboratoires Beaufour, UCB-Pharma, Lambdatech, Lipha, IPSEN, Zyma, Madaus Pharma, Servier.

Scientific Grants and Research contracts

14 contracts with the FNRS, FRFC,IRSIA, and Région Wallonne

PROFESSIONAL AND SCIENTIFIC ASSOCIATIONS

Member of 15 scientific societies
Member of 33 Ph.D. thesis jurys
Member of the research comitte for Biomed 1 and 2 of the EEC

PRESENTATIONS OF RESULTS IN SCIENTIFIC CONGRES

171.presentations in scientific meetings as author or co-author.

INVITED OR PLENARY CONFERENCES OR LECTURES.

75 presentations in scientific meetings under invitation

MAIN SUBJECTS OF RESEARCH

Cellular Ageing and modelisation of the ageing process using the thermodynamics of open systems; the role of Free radical and the importance of the antioxidant enzymes.

Study of endothelial cells under hypoxia in correlation with the development of varicose diseases

Development of new diagnostic assays using bioluminescence: ELISA, DNA probes for virus and bacteria detection.

PUBLICATIONS

The author's scientific output consists of 125 research papers in peer-reviewed international journals